Lebenslauf

Name	Popp, Alexandra Muriel
Geburtsdatum	31. Mai 1976
Geburtsort	Münsterlingen TG
Nationalität	Schweiz
Heimatort	Steinach SG

1983 - 1989	Primarschule in Kreuzlingen
1989 - 1992	Sekundarschule in Kreuzlingen
1992 - 1996	Kantonsschule in Kreuzlingen
1996	Matura Typus C
1996 - 1998	Studium der Veterinärmedizin an der Vetsuisse-Fakultät Universität Zürich, Schweiz
2003 - 2007	Studium der Veterinärmedizin an der Vetsuisse-Fakultät Universität Zürich, Schweiz
2007	Staatsexamen Veterinärmedizin an der Vetsuisse-Fakultät Universität Zürich, Schweiz
2000 - 2002	Sachbearbeiterin Documentation Management Cilag AG, Schaffhausen
2003	Assistentin/Sekretärin Leibundgut Partner AG, Ermatingen
2004 - 2007	Teilzeit Mitarbeiterin Schweizer Tierarzneimittelkompendium, Institut für Veterinärpharmakologie und -toxikologie der Vetsuisse-Fakultät Universität Zürich, Schweiz
2008 - 2009	Doktorandin, Institut für Lebensmittelsicherheit und -hygiene der Vetsuisse-Fakultät Universität Zürich, Schweiz

19. März 2010

Institut für Lebensmittelsicherheit und -hygiene
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. Roger Stephan

Identification and further characterization of *Enterobacteriaceae* strains isolated from an infant formula processing plant

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Alexandra Muriel Popp

Tierärztin
von Steinach SG, Schweiz

genehmigt auf Antrag von

Prof. Dr. Roger Stephan, Referent

Prof. Dr. Peter Kuhnert, Korreferent

Zürich 2010

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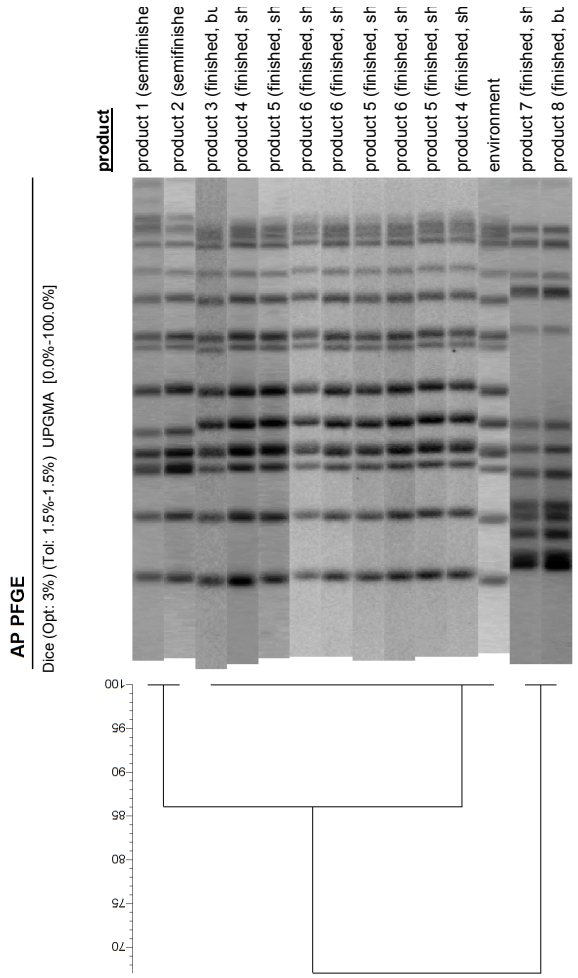

Fig 2. Comparison of band patterns of three groups of *E. cloacae* isolates calculated with the GelCompar means different kind of products, which means different brand names. Identical product numbers means th identical product.

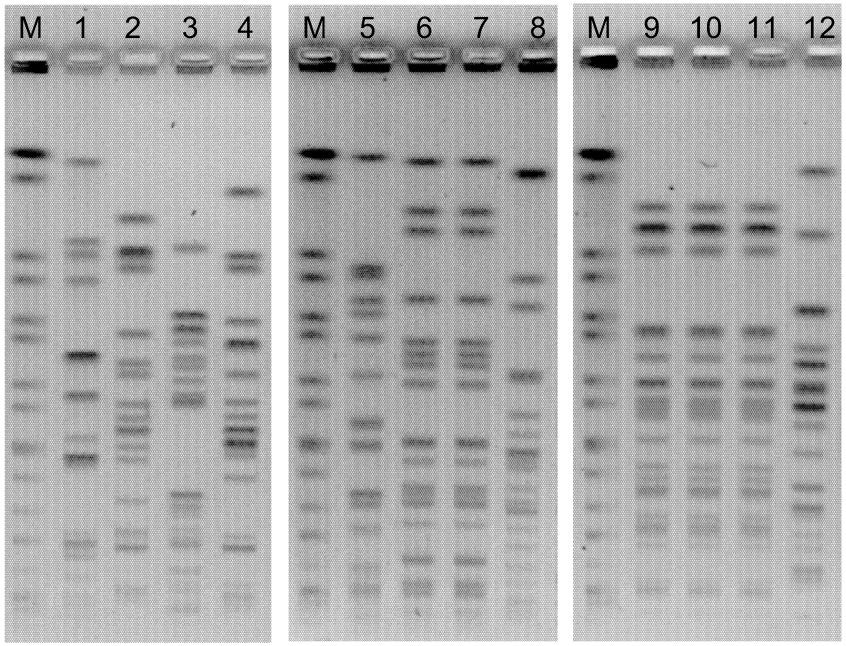

Fig 1. PFGE patterns of chromosomal DNA restriction fragments digested with *Xba*I resolved in 1% pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer.
M, molecular size standard (*Salmonella* Braenderup strain H9812 digested with *Xba*I); lines 1-4 isolates of *E. cloacae*; lines 5-8, isolates of *L. adecarboxylata*; lines 9-12 isolates of *K. pneumoniae*.

Identification and further characterization of *Enterobacteriaceae* strains isolated from an infant formula processing plant

Alexandra Muriel Popp, 2010
Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich
Contact: Institute for Food Safety and Hygiene (Secretariat), ils@fsafety.uzh.ch,
Tel. +41-44-635-8651

Summary

Over the past few years the group of *Enterobacteriaceae* has considerably gained in importance for food industries. The aim of this study was to identify *Enterobacteriaceae* isolates from different sample types of an infant formula processing plant and to genotype frequently isolated species in order to trace back contamination routes. A total of 470 isolates originating from raw ingredients (n = 117), environmental samples (n = 166) and finished products (n = 187) were analyzed by biochemical tests as well as by *rpoB* sequencing. The most common species was *Enterobacter cloacae* (n = 161), followed by *Pantoea* spp. (n = 51) and *Klebsiella pneumoniae* (n = 39). Furthermore, two novel *Pantoea* spp. (*Pantoea gaviniae* und *Pantoea calida*) could be described in the frame of this study. A total of 216 strains from the species *Enterobacter cloacae* (n = 155), *Klebsiella pneumoniae* (n = 37) and *Leclercia adecarboxylata* (n = 24) were selected for genotyping. Restriction digest with *Xba*I revealed discriminative PFGE patterns for all three species. Heat sensitive additives could be traced back as contamination source for products which is comparable to the situation described for *Cronobacter* spp. Especially *E. cloacae* that can apparently be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for hygiene monitoring along the processing chain.

Zusammenfassung

Während der letzten Jahre haben *Enterobacteriaceae* in der Lebensmittelindustrie zunehmend an Bedeutung gewonnen. Das Ziel dieser Arbeit war die Identifizierung von *Enterobacteriaceae* aus verschiedenen Kompartimenten eines Produktionsbetriebes für Säuglingsanfangsnahrung und die Genotypisierung von häufig gefundenen Spezies um mögliche Kontaminationswege aufzuzeigen. Insgesamt 470 Isolate aus Rohmaterial (n = 117), Umgebungsproben (n = 166) und Endprodukten (n = 187) wurden mittels biochemischenr Tests sowie *rpoB* Sequenzierung identifiziert. Am häufigsten gefunden wurde *Enterobacter cloacae*, gefolgt von *Pantoea* spp. (n = 51) und *Klebsiella pneumoniae* (n = 39). Zudem konnten im Rahmen dieser Arbeiten zwei neue *Pantoea* spp. (*Pantoea gaviniae* und *Pantoea calida*) beschrieben werden. Zur Genotypisierung wurden *Enterobacter cloacae* (n = 155), *Klebsiella pneumoniae* (n = 37) und *Leclercia adecarboxylata* (n = 24) ausgewählt. Ein Restriktionsverdau der insgesamt 216 Stämme mit *XbaI* ergab aussagekräftige Bandenmuster für alle drei Spezies. Hitzelabile Zusatzstoffe konnten als mögliche Kontaminationsquelle für Endprodukte identifiziert werden, was für *Cronobacter* spp. ähnlich beschrieben ist. Besonders *E. cloacae*, der offensichtlich in den selben Nischen wie *Cronobacter* spp. aber häufiger vorkommt, könnte daher für ein prozessbegleitendes Hygienemonitoring verwendet werden.

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different dates. This possibly indicates a persistent contamination of the facilities. Since only dry cleaning is practicable in this kind of production environment, disinfectants are not efficient. Furthermore dust formation can not totally be avoided and therefore air flow management is very important.

An other possible way of contamination is shown by the following example. One group (fig. 2) showed the same genotype of *E. cloacae* isolates from products that were manufactured in the two different production sites, 100 km apart, so the environment as possible source of contamination could be excluded. Checking the ingredients of those various products made evident that they all contained lactose, which is a carrier for heat sensitive additives, and therefore added after the drying process, from the same supplier. This indicates heat sensitive additives as an important source of entry into the production facilities.

To summarize, the findings are comparable with the situation described for *Cronobacter* spp. (Mullane et al., 2007; Iversen et al., 2009). Especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for a hygiene monitoring program along the processing chain in order to trace back contamination routes. Certain genotypes of *E. cloacae*, that are able to persist within the factory environment, might possess special properties as e.g. enhanced desiccation tolerance and therefore demonstrate the need for further evaluation of stress response mechanisms in this species.

List of publications

I. **Identification of *Enterobacteriaceae* isolates from raw ingredients, environmental samples and products of an infant formula processing plant**
Popp A, Iversen C, Fricker-Feer C, Gschwend K, Stephan R
Arch Lebensmittelhyg 2009, 60(3):92-97.

II. ***Pantoea gaviniae*, sp. nov. and *Pantoea calida*, sp. nov. isolated from infant formula and infant formula production environment**
Popp A, Cleenwerck I, Iversen C, De Vos P, Stephan R
Int J Syst Evol Microbiol 2010, in press.

III. **PFGE-typing of *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Leclercia adecarboxylata* isolates from an infant formula processing plant**
Popp A, Fricker-Feer C, Gschwend K, Stephan R
Arch Lebensmittelhyg 2010, in press.

Archiv für Lebensmittelhygiene 60, Heft 3 (2009), Seiten 92–97

Arch Lebensmittelhyg 60, 92–97 (2009)
DOI 10.2376/0003-925X-60-92

ISSN 0003-925X

Korrespondenzadresse:
stephanr@fsafety.uzh.ch

Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland[1]
Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland[2]
QA and Food Safety Department Hochdorf Nutritec AG, 6280 Hochdorf, Switzerland[3]

Identification of *Enterobacteriaceae* isolates from raw ingredients, environmental samples and products of an infant formula processing plant

Identifizierung von Enterobacteriaceae Isolaten aus verschiedenen Proben eines Produktionsbetriebes für Säuglingsanfangsnahrung

Alexandra Popp[1], Carol Iversen[1,2], Claudia Fricker-Feer[3], Karl Gschwend[3], Roger Stephan[1]

Summary

Over the past few years the group of *Enterobacteriaceae* has considerably gained in importance for food industries, especially for infant formula processing plants. Up to now, only a few studies have focused on the diversity of this group in powdered infant formula and related products. Therefore, the aim of this study was to identify *Enterobacteriaceae* isolates from a previous study from different samples of an infant formula processing plant. A total of 470 isolates from raw ingredients (n = 117), environmental samples (n = 166) and finished products (n = 187) were further identified by biochemical tests as well as by *rpoB* sequencing. The most common species was *Enterobacter cloacae* (n = 161), followed by *Pantoea* spp. (n = 51) and *Klebsiella pneumoniae* (n = 39). In total, 65 isolates could not be identified. Using the partial *rpoB* gene sequence data as a similarity matrix, 22 different clusters could be formed within these 65 isolates. The fact that some isolates from the same *rpoB* cluster originate from different sample types might indicate that they are widely common within such a production environment and hence of practical relevance. These isolates which form possibly novel genus/species will be further investigated.

Keywords: powdered infant formula, infant food, *Enterobacteriaceae*

Zusammenfassung

Im Verlauf der letzten Jahre hat die Bedeutung der Gruppe der *Enterobacteriaceae* in der Lebensmittelindustrie, speziell in Produktionsbetrieben für Säuglingsanfangsnahrung, deutlich zugenommen. Bis zum heutigen Zeitpunkt wurden nur wenige Studien durchgeführt, die schwerpunktmäßig die Diversität dieser Gruppe in Säuglingsanfangsnahrung in Pulverform und ähnlichen Produkten untersuchten. Demzufolge war das Ziel dieser Studie, *Enterobacteriaceae* Isolate, die in einer vorhergehenden Untersuchung aus verschiedenen Proben aus einem Produktionsbetrieb für Säuglingsanfangsnahrung isoliert wurden, näher zu identifizieren. Insgesamt wurden 470 Isolate aus Ausgangsmaterialien (n = 117), Umgebungsproben (n = 166) und Endprodukten (n = 187) mittels biochemischer Tests sowie *rpoB* Sequenzierung näher identifiziert. Die am häufigsten nachgewiesene Spezies stellte *Enterobacter cloacae* dar (n = 161), gefolgt von *Pantoea* spp. (n = 51) und *Klebsiella pneumoniae* (n = 39). Insgesamt konnten 65 Isolate nicht identifiziert werden. Unter Verwendung von *rpoB* Gensequenzdaten als Ähnlichkeitsmatrix konnten 22 verschiedene Cluster innerhalb dieser 65 Isolate gebildet werden. Die Tatsache, dass manche Isolate aus demselben *rpoB* Cluster aus verschiedenen Probentypen stammten, könnte darauf hinweisen, dass sie in derartigen Produktionsstätten weit verbreitet und somit von praktischer Bedeutung sind. Diese Isolate, welche möglicherweise neue Gattungen oder Arten darstellen, werden noch genaueren Untersuchungen unterzogen werden.

Schlüsselwörter: Säuglingsanfangsnahrung, *Enterobacteriaceae*

Rad, Hercules, CA) from which tagged image file format (TIFF) files were imported into GelCompar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Clustering of the PFGE fingerprint patterns was performed using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA), with an optimization of 3% and band position tolerance of 1.5%. Cophenetic correlations ranged from 61-100% and the relatedness of the fingerprint patterns was compared at 95% similarity.

Isolates with identical band patterns were considered as a group.

Results and Discussion

Restriction digest with *Xba*I revealed discriminative PFGE patterns for all three species. *E. cloacae* showed patterns consisting of 10-20 bands, *K. pneumoniae* had patterns with 11-20 bands and the patterns for *L. adecarboxylata* contained 11-19 bands (fig. 1). In summary no difference in discriminative power could be observed between the three species, indicating that *Xba*I was appropriate for all of them.

The 155 *E. cloacae* isolates showed 92 different pulso-types, allocated in 24 groups with 2-11 isolates each. 17 groups contained only isolates from product samples, 3 groups only environmental samples. The other 4 groups contained isolates from different sample types. For *K. pneumoniae* there were 37 isolates resulting in 25 different pulso-types. 5 groups containing 2-6 isolates were formed, all containing product samples only. The 25 *L. adecarboxylata* isolates resulted in 21 different pulso-types with 2 groups. One of them contained 2 isolates from raw ingredients, the other contained 3 isolates from product and environmental samples.

Various groups, especially for *E. cloacae,* were formed by isolates from different kind of finished products. A closer look at these products revealed that they were either produced on the same spray dryer, blended in the same mixer or packed in the same filling line. In other cases groups contained isolates from the same kind of finished product but manufactured on

Pulsed-Field Gel Electrophoresis

Isolates were grown on blood agar overnight at 37°C. Bacteria were harvested from the plate using a cotton swab and transferred into 2 ml cell suspension buffer (100 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0). The suspension was adjusted spectrophotometrically to an optical density of 1.0 at 600 nm. 400 µl of this suspension was mixed with 20 µl of Proteinase K solution (20 mg/ml) and 400 µl of 1.4% (w/v) pulsed-field certified agarose (Bio-Rad Laboratories AG, Reinach BL, Switzerland). Agarose plugs were prepared in plug moulds (Bio-Rad) and allowed to solidify. Then plugs were placed into tubes containing 5 ml lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1% (w/v) N-Laurylsarcosine) with addition of 0.1 mg/ml Proteinase K immediately prior to use. After incubation at 55°C overnight the plugs were washed with distilled water twice and with TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) four times with incubation time of 30 min and 60 min respectively at 50°C. The washed plugs were stored in TE buffer at 4°C. For restriction endonuclease digestion, agarose plugs were cut in 2 mm slices and equilibrated in 300 µl restriction buffer H (Roche) for 20 min. The restriction digest was performed in 300 µl fresh restriction buffer H containing 80 U of enzyme *Xba*I (Roche) for 12-14 h. Restriction fragments were separated in a 1% (w:v) pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer with a CHEF-DR III system (Bio-Rad). The running buffer was supplemented with 50 mM Thiourea (Sigma); this overcomes the problem of non-typeable strains due to the nucleolytic activity of a peracid derivative of Tris, which can form at the anode during electrophoresis (Ray et al., 1995). *Salmonella* Braenderup strain H9812 digested with *Xba*I was used as a molecular size standard (Hunter et al., 2005). The following conditions were used for the separation of the digested fragments, pulse time: 5-50 sec, linear ramping for 20 h at 14°C, 120° included angle. Following electrophoresis, gels were stained for 30 min with ethidium bromide (5 mg/l) and destained in distilled water for 1 minute. Fingerprint patterns were visualized and captured using a CCD photography system (Bio-

Introduction

In Regulation (EC) 2073/2005 (Anonymous, 2005) the European Commission states that *Salmonella* and *Cronobacter* spp. are the microorganisms of greatest concern in powdered infant formula. The presence of these pathogens constitutes a considerable risk when conditions allow growth of the bacteria. In particular, *Cronobacter* spp. are known to cause infections in neonates with severe outcomes such as sepsis, meningitis or necrotizing enterocolitis due to contaminated infant formula (Biering et al., 1989; Bar-Oz et al., 2001; van Acker et al., 2001; Himelright et al., 2002).

Enterobacteriaceae can be used as process hygiene criteria for various products. Notably for powdered infant formula the tolerance levels are low (Anonymous, 2005). Up to now, only a few studies have focused on the diversity of this group in infant formula (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Since *Enterobacteriaceae* should not survive the heating processes used during manufacture, a recontamination of the powdered infant formula after this process step must be assumed. Thus, also the presence and diversity of *Enterobacteriaceae* in the production environment and in raw ingredients is of interest. The aim of this study was therefore to identify *Enterobacteriaceae* isolates other than *Cronobacter* spp. and *Salmonella*, which were gained from different sample types (raw ingredients, environment, finished products) of an infant formula processing plant.

Material and Methods

Isolates

A total of 470 *Enterobacteriaceae* isolates (117 isolates from raw ingredients; 166 isolates from environmental samples; 187 isolates from finished products) were gained from an infant formula processing plant within a previous study (Iversen et al., 2009). The raw ingredients comprised milk, flours, starches, sugars, fruit and vegetable flakes and powders, vitamins and minerals, flavorings and emulsifiers. The environmental samples covered the manufacturing process including filling and storage equipment, walls, floors, handles, filters, cleaning equipment, dust, sinks, elevators and personnel. The finished products incorporated a range of food brands for infants of all ages and nutritional needs.

TABLE 1: *Identification of 470 isolates from different samples of an infant formula processing plant by API ID32E and rpoB sequencing*

	Raw ingredient			Product			Environment		
Genus, species	A[1]	S[2]	T[3]	A[1]	S[2]	T[3]	A[1]	S[2]	T[3]
Butiauxella agrestis	1	–	1	–	–	–	–	–	–
Citrobacter amalonaticus/farmeri	–	–	–	1	–	1	2	–	2
Citrobacter braakii	–	–	–	–	–	–	3	–	3
Citrobacter freundii	–	–	–	–	–	–	6	–	6
Enterobacter cloacae	23	7	30	72	23	95	22	14	36
Enterobacter cancerogenus	3	–	3	8	–	8	–	–	–
Enterobacter pulveris	–	4	4	–	2	2	–	–	–
Enterobacter helveticus	–	2	2	–	1	1	–	8	8
Enterobacter amnigenus	1	–	1	1	–	1	2	–	2
Enterobacter asburiae	–	1	1	–	–	–	–	–	–
Enterobacter cowanii	–	2	2	–	–	–	–	–	–
Enterobacter turicensis	–	3	3	–	–	–	–	–	–
Escherichia coli	3	–	3	8	–	8	8	1	9
Escherichia hermannii	3	–	3	6	–	6	7	–	7
Escherichia vulneris	1	–	1	–	–	–	4	–	4
Hafnia alvei	2	–	2	–	–	–	1	–	1
Klebsiella pneumoniae	4	–	4	27	1	28	6	1	7
Klebsiella oxytoca	2	2	4	10	2	12	7	4	11
Klebsiella singaporensis	–	–	–	–	–	–	–	1	1
Klebsiella variicola	–	1	1	–	–	–	–	–	–
Leclercia adecarboxylata	11	–	11	6	–	6	10	–	10
Pantoea spp.	26	–	26	11	–	11	13	1	14
Raoultella terrigena	1	1	2	–	–	–	–	–	–
Serratia nematodiphila	–	–	–	–	–	–	–	1	1
Unknown	–	13	13	–	8	8	–	44	44

[1] Isolates that could be identified with API ID32E.

[2] Isolates with insufficient API results where *rpoB* sequencing had to be done in addition.

[3] Total.

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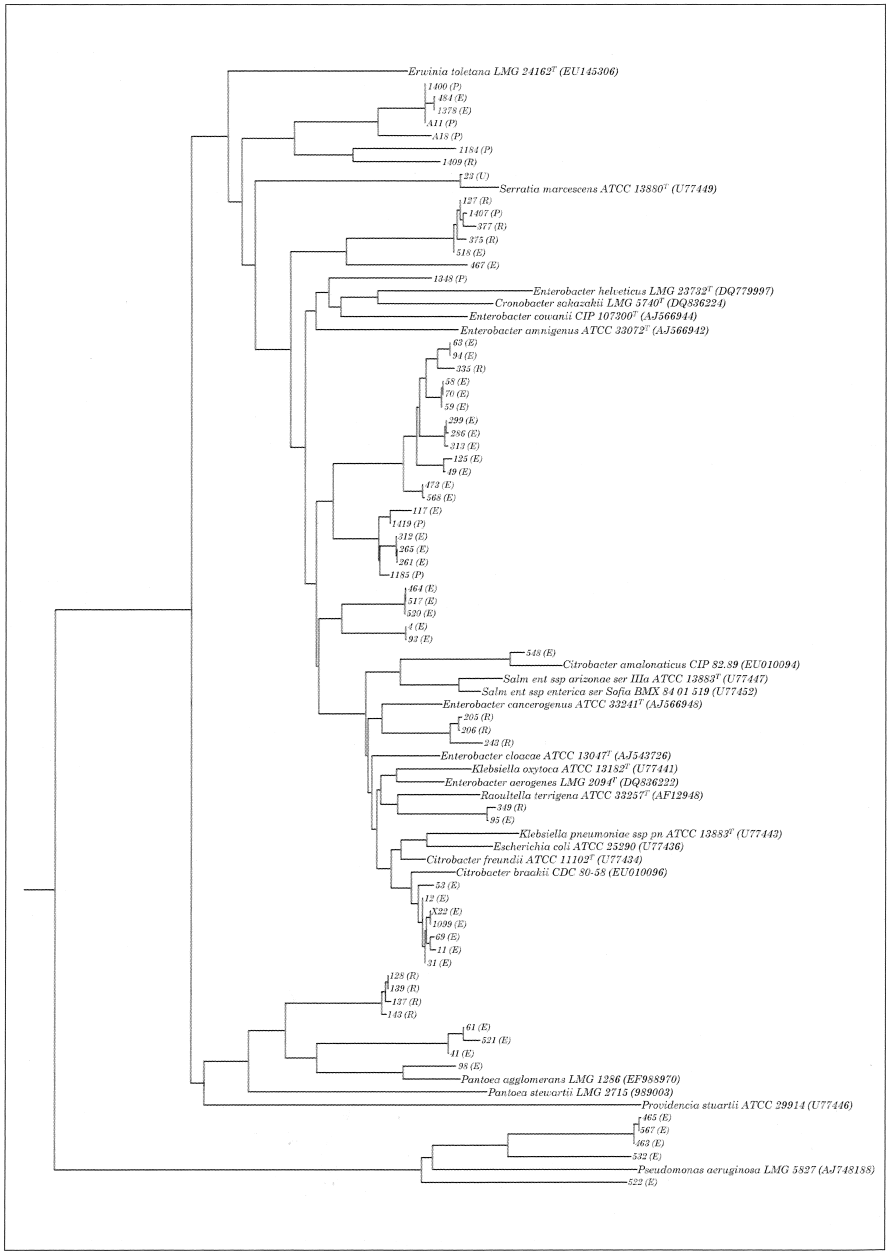

FIGURE 1: *Neighbour-joining tree of rpoB gene sequences showing the estimated phylogenetic relationships between unidentified isolates and reference strains. Constructed with ClustalW (on http://align.genome.jp/) with the following multiple alignment parameters: gap open penalty: 15, gap extension penalty: 6.66, weight transition: no, Hydrophilic Gaps: yes, Selected Weight Matrix: IUB. Origin of the unidentified isolates in brackets. R = raw ingredients, P = finished products, E = environment.*

Materials and Methods

Manufacturing facility

The firm is one of the leading milk processing companies in Switzerland and has two milk-associated production sites (site A, site B) about 100 km apart. It handles about 350'000 tons of milk per year and fabricates various products like milk powder, powdered infant formula and follow on and growing up formula that are sold in over 60 countries. Customers include the food industry, the retail industry, bakeries and the catering trade.

Isolates

Isolates were collected within a previous study (Popp et al., 2009) as different sample types from the two different production sites. Sample types were raw ingredients (for example milk, flours, starches, sugars, vitamins and flavorings), environmental samples (for example filling equipment, walls, floors, cleaning equipment, dust, sinks, elevators and personnel) and product samples. Product samples were taken from different steps of manufacturing and can be differentiated between semifinished products (before adding supplements) and finished products. Finished products were powdered infant formula and follow on formula from different brands that can be bulk goods or shelf-ready products.

A total of 216 isolates from three different species were selected for genotyping. From the 155 *Enterobacter cloacae* isolates 25 were from raw ingredients, 16 from the environment and 94 from products. From the 37 *Klebsiella pneumoniae* isolates 4 were from raw ingredients, 6 from the environment and 27 from products. From the 24 *Leclercia adecarboxylata* isolates 11 were from raw ingredients, 7 from the environment and 6 from products.

Introduction

The family of *Enterobacteriaceae* is a useful indicator for a hygiene monitoring system in food production facilities and food products. For powdered infant formula the absence of *Enterobacteriaceae* in 100 g (n=10; M=absence in 10 g) is required (Anonymous, 2005). Nevertheless, occasionally *Enterobacteriaceae* can be detected in infant formula and production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006; Popp et al., 2009). On one hand there are supplements that need to be added after the drying process since they are not heat stable, and on the other hand during the filling process contamination is possible while the system is not completely close.

Up to now, few studies have focused on the presence and the diversity of *Enterobacteriaceae* in infant formula and the related production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Two recent surveys concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in powdered infant formula (PIF) manufacturing facilities in order to investigate clonal persistence and identify possible dissemination routes along the processing chain by the use of PFGE (Mullane et al., 2007; Iversen et al., 2009). Identical pulso-types of strains isolated from different locations indicated that the production environment is an important source for product contamination.

In a previous study (Popp et al., 2009) 470 *Enterobacteriaceae* isolates from different sample types of a Swiss infant formula processing plant were identified. Frequently isolated species were *Enterobacter cloacae* (34%), *Klebsiella pneumoniae* (8%) and *Leclercia adecarboxylata* (6%). The aim of this study was to genotype these isolates in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

Identification

Biochemical identification

For this study, only isolates which could not be identified in the previous study (Iversen et al., 2009) as *Cronobacter* spp. were further analyzed. All isolates were identified with API ID 32E (bioMérieux (Suisse) SA, Genève, Switzerland) according to the manufacturer's protocol. For each isolate with an identification result below „good" a partial *rpoB* gene sequence analysis was performed in addition.

rpoB sequencing

Bacterial DNA was extracted by boiling the colonies. PCR amplifications were performed in 50 µl reaction tubes containing the forward primer CM_7 (5'-aac cag ttc cgc gtt ggc ctg g-3') and reverse primer CM_{31b} (5'-cct gaa caa cac gct cgg a-3') (Mollet et al., 1997) at 20 pmol each by using the GoTaq Green Master Mix (Promega, Madison, WI, USA). The PCR conditions were 90 s at 94°C followed by 40 cycles at 94°C for 10 s, 55°C for 20 s and 72°C for 50 s, completed by a final elongation step at 72°C for 5 min (Mollet et al., 1997). PCR products were resolved on a 1% agarose gel stained with ethidium bromide and examined under UV light. After purification with the PCR purification kit (Qiagen, Hombrechtikon, Switzerland), sequencing reactions were performed by Microsynth AG (Balgach, Switzerland). The obtained sequences were compared with the nucleotide collection of NCBI using the nucleotide BLAST optimized for highly similar sequences. With a similarity above 98% the isolate was assigned to the corresponding species. Isolates with a similarity less than 98% were considered as unidentified and hence as potential novel species.

rpoB neighbour-joining tree

The *rpoB* sequences of these unidentified isolates were compared to those of 22 reference strains from the NCBI nucleotide database using ClustalW on http://align.genome.jp/. The *rpoB* neighbour-joining tree was calculated with the following multiple alignment parameters: gap open penalty: 15, gap extension penalty: 6.66, weight transition: no, Hydrophilic Gaps: yes, Selected Weight Matrix: IUB.

Results and Discussion

The most common species out of all 470 isolates we identified were 161 *Enterobacter* (*Eb.*) *cloacae* (34%), followed by 51 *Pantoea* spp. (11%) and 39 *Klebsiella* (*K.*) *pneumoniae* (8%). If considering only the 187 isolates from finished products, there were 95 *Eb. cloacae* (50%), 28 *K. pneumoniae* (15%) and 11 *Pantoea* spp. (6%) (Tab. 1).

Of the total of 470 isolates 322 (69%) could be identified with API ID32E to adequate quality, for 148 isolates (31%) *rpoB* sequencing was needed. We chose the *rpoB* gene which is known to produce more reliable outputs compared to sequencing the *16S* rRNA gene (Mollet et al., 1997; Case et al., 2006; Brady et al., 2008; Paauw et al., 2008).

From the 148 isolates which were sequenced, 83 isolates (56%) showed the demanded *rpoB* gene similarity and could thus be assigned to an existing species, whereas 65 isolates (44%) could not be identified. From these 65 isolates 13 were found in raw ingredients, 44 in environmental samples and 8 in finished products. Using the partial *rpoB* gene sequence data as a similarity matrix 22 different clusters could be formed. These clusters contained one to 13 different isolates originating either from just one or up to all three sample types (Fig. 1). The fact that some isolates from the same *rpoB* cluster originate from different sample types (raw ingredients, environment and finished products) might indicate that they are widely common within this production environment and hence of practical relevance. These isolates which possibly form novel genus/species will be further investigated.

Muytjens et al. (1988) has been the first group that described the quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. They found *Eb. agglomerans* (now *Pantoea* [*P.*] *agglomerans*) with 27% of all isolates, to be the most common species, followed by *Eb. cloacae* with 23% and *K. pneumoniae* with 10%. However, in this study the identity of *Eb. agglomerans* is uncertain because since then the *Enterobacter-Pantoea-Citrobacter* group has been revised. Iversen and Forsythe (2004), looking at powdered infant formula milk and related products, described a distribution of 25% *Eb. cloacae*, 19% *P. agglomerans* and 2% *K. pneumoniae*. Recently, Estuningsih et al. (2006) found 24% *P. agglomerans*, 20% *Escherichia hermanii*, 16% *Eb. cloacae* and 6% *K. pneumoniae* in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. Compared to these results, we found higher numbers of *Eb. cloacae*.

Eb. cloacae is described as an emerging pathogen in neonatal care units causing mostly sepsis (Tresoldi et al., 2000; van Dijk et al., 2002; Talon et al., 2004; Dalben et al., 2008). Only rare cases of necrotizing enterocolitis were published (Powell et al., 1980; van Nierop et al., 1998). In a recently published review sixteen *Eb. cloacae* sepsis outbreaks were described (Dalben et al., 2008). In 50% of these outbreaks the source of infection could not be found. In other cases different sources were mentioned, like contaminated parenteral nutrition solution, ther-

mometers or hands of personnel (Shi et al., 1996; Tresoldi et al., 2000; van Dijk et al., 2002). In another study the gastrointestinal tracts of the patients and environmental surfaces have been assumed as principal sites of bacterial reservoir (Yu et al., 2000).

Pantoea spp. are actually known as plant pathogens (Coutinho et al., 2002; Cother et al., 2004; Goszczynska et al., 2007), which might explain the comparatively high number found in raw ingredients. In recent years, *Pantoea* strains have been consistently linked with human infections (Cruz et al., 2007). Van Rostenberghe et al. (2006) described eight cases of neonates with respiratory distress syndrome caused by *Pantoea* spp. where the source of infection was contaminated parenteral nutrition solution. Two outbreaks of sepsis have been reported, either caused by contaminated parenteral nutrition solution or by a contaminated tranference tube (Habsah et al., 2005; Bicudo et al., 2007).

Klebsiella is known as a health care associated pathogen (Gaillot et al., 1998; Cassettari et al., 2009). The urinary tract is the most common site of infection (Podschun and Ullmann, 1998). But also septicaemia, respiratory distress, meningitis or conjunctivitis have been described (Gaillot et al., 1998; Persson et al., 2002; Cassettari et al., 2009). Furthermore, outbreaks of extended-spectrum beta-lactamase producing *K. pneumoniae* in neonatal intensive care units have been reported (Gaillot et al., 1998; Macrae et al., 2001; Gupta et al., 2004; Cassettari et al., 2009). This creates considerable difficulty when empiric antibiotic therapy is needed for seriously ill neonates. Health care workers may act as a vehicle in the transmission between patients (Gupta et al., 2004; Cassettari et al., 2009), but situations in which there was a common source of infection have also been reported (Cassettari et al., 2009). The gastrointestinal tract of asymptomatic patients can serve as reservoir for this pathogen (Gupta et al., 2004), this is also confirmed by another study where 27 of 120 asymptomatic newborn infants were found to be colonized (Cassettari et al., 2009).

Although these frequently isolated species are known as pathogens for neonates, no correlation with contaminated infant formula has been described so far. However, these widely spread species might be used in an infant formula processing plant as indicator microorganisms to identify and trace back contamination routes.

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Abstract

The family of *Enterobacteriaceae* is a useful indicator for hygiene conditions in food production facilities and food products. For powdered infant formula (PIF) the absence of *Enterobacteriaceae* in 100 g is required. Nevertheless, occasionally *Enterobacteriaceae* can be detected. A recent study concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in PIF manufacturing facilities in order to investigate clonal persistence and identify possible transmission routes. The aim of this study was to genotype isolates from frequently found other species within the family of *Enterobateriaceae* in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

In total 216 isolates from three different species (*Enterobacter (E.) cloacae*, *Klebsiella (K.) pneumoniae* and *Leclercia (L.)adecarboxylata*) were genotyped. The isolates originated from raw ingredients, environment and products of an infant formula processing plant. Restriction digest with *Xba*I revealed discriminative PFGE patterns consisting of 10-20 bands for all three species. Heat sensitive additives could be traced back as contamination source for products. Furthermore, the production environment was found as a reservoir for persisting strains. Showing analogy to the situation described for *Cronobacter* spp., especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for hygiene monitoring along the processing chain. Certain genotypes of *E. cloacae*, that are able to persist within the factory environment, might possess special properties as e.g. enhanced desiccation tolerance enabling them to survive the harsh environmental conditions.

PFGE-typing of *Enterobacter cloacae, Klebsiella pneumoniae* and *Leclercia adecarboxylata* isolates from an infant formula processing plant

Alexandra Popp[1], Claudia Fricker-Feer[2], Karl Gschwend[2], Roger Stephan[1]*

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland.

[2] QA and Food Safety Department Hochdorf Nutritec AG, CH-6280 Hochdorf, Switzerland.

*Corresponding author. Mailing address: Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland. Phone: +41-44-635-8651. Fax: +41-44-635-8908. E-mail: stephanr@fsafety.uzh.ch

Keywords: PFGE, Genotyping, *Enterobacteriaceae*, infant food

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Address for correspondence:
Prof. Dr. Roger Stephan
Institute for Food Safety and Hygiene
Vetsuisse Faculty University of Zurich
Winterthurerstrasse 272
8057 Zurich
Switzerland
stephanr@fsafety.uzh.ch

Pantoea gaviniae, sp. nov. and *Pantoea calida*, sp. nov. isolated from infant formula and infant formula production environment

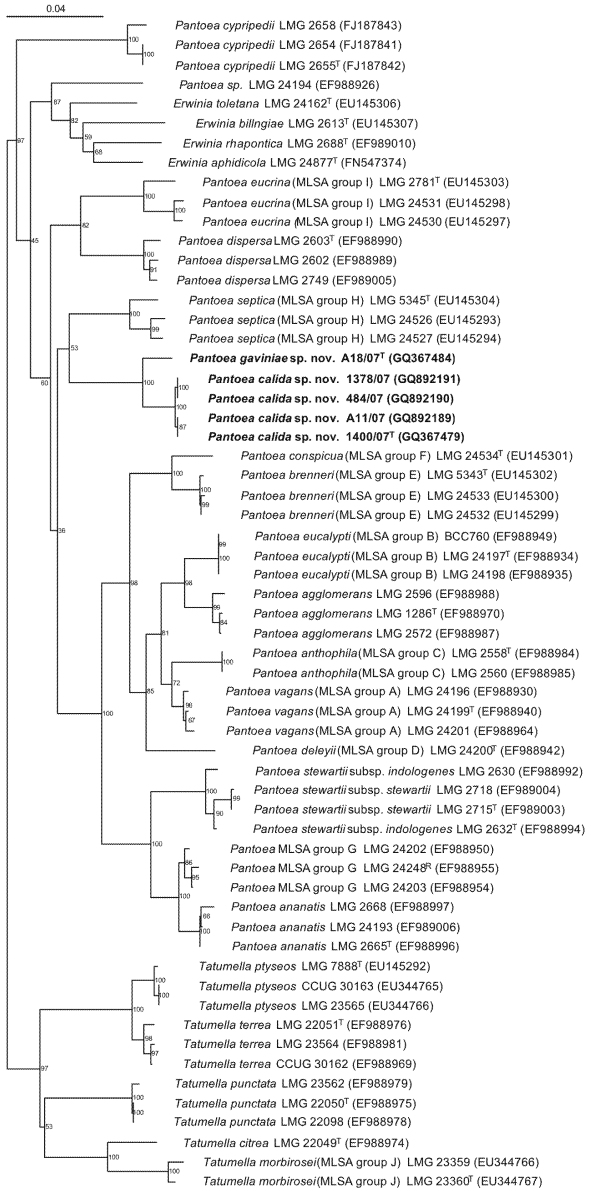
Alexandra Popp[1], Ilse Cleenwerck[2], Carol Iversen[3], Paul De Vos[2] and Roger Stephan[1]*

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland

[2]BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35, 9000 Gent, Belgium

[3]Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

*Corresponding author. Mailing address: Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland. Phone: +41-44-635-8651. Fax: +41-44-635-8908. E-mail: stephanr@fsafety.uzh.ch



Supplementary figure 1: Neighbour-joining dendrogram based on *rpoB* gene sequences showing the phylogenetic relationships between *P. gaviniae* sp. nov., *P. calida* sp. nov., and related taxa within the family *Enterobacteriaceae*. The type strains are indicated with ^{T}. The reference strains of MLSA groups reported by Brady et al. (2008) are indicated with ^{R}. The scale bar indicates 4 % nucleotide substitutions. Bootstrap values (percentages of 1000 replicates) are shown.

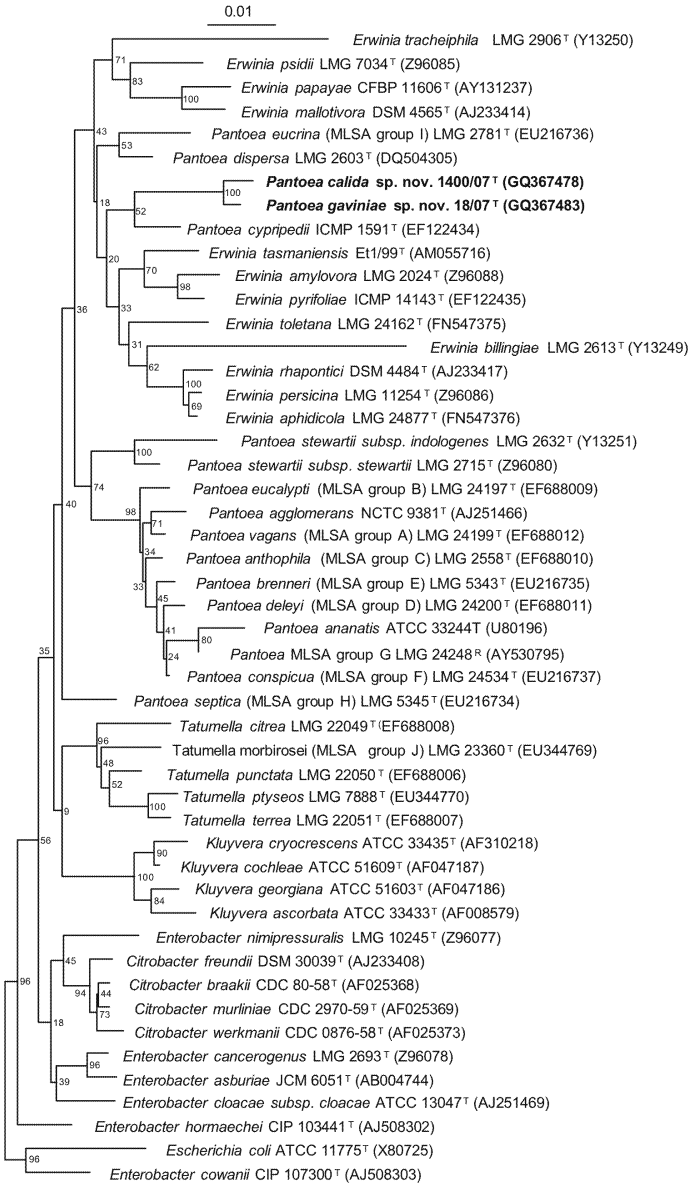


Figure 2: Neighbour-joining dendrogram based on 16S rRNA gene sequences showing the phylogenetic relationships between *P. gaviniae* sp. nov., *P. calida* sp. nov. and related taxa within the family *Enterobacteriaceae*. The type strains are indicated with ^{T}. The reference strains of MLSA groups reported by Brady et al. (2008) are indicated with ^{R}. The scale bar indicates 1 % nucleotide substitutions. Bootstrap values (percentages of 1000 replicates) are shown.

SUMMARY

Five isolates, Gram-negative, facultative anaerobic, non-spore-forming coccoid rods, were obtained from infant formula and infant formula production environment and investigated in a polyphasic taxonomic study. Biochemical tests and partial *rpoB* gene sequence analysis of the five isolates revealed that they formed two distinct groups in the family *Enterobacteriaceae*, closely related with several *Pantoea* spp. and *Erwinia* spp, which indicated a phylogenetic position in the genus *Pantoea* or the genus *Erwinia*. Multilocus sequence analysis of concatenated partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences of two representatives, suggested that they constituted probably two novel species in the genus *Pantoea*, phylogenetically closely related to *Pantoea septica*. The five isolates showed the general characteristics of the genus *Pantoea*, and DNA-DNA hybridizations between two representatives and the type strains of the phylogenetically closest relatives according to comparative 16S rRNA sequence analysis, showed that the isolates represented two novel genospecies. These two genospecies could be differentiated from each other by fermentation of galactonurate, sorbitol and Potassium-5-ketogluconate. From the phylogenetically related *Pantoea* species they could be distinguished by their ability to ferment D-lactose and utilize β-gentiobiose and D-raffinose, their inability to ferment and utilize D-arabitol and their inability to produce indol.

On the basis of the obtained results it is proposed to classify the isolates as *Pantoea gaviniae,* sp. nov. (type strain A18/07^{T} = LMG 25382^{T} = DSM 22758^{T}) and *Pantoea calida*, sp. nov. (four isolates, type strain 1400/07^{T} = LMG 25383^{T} = DSM 22759^{T}).

Over the past few years, the family *Enterobacteriaceae* has gained in importance for food industries, especially for infant formula processing plants. During a recent study 470 *Enterobacteriaceae* isolates from raw ingredients, environmental samples and products of an infant formula processing plant were investigated by biochemical tests (API ID32E) and *rpoB* gene sequence analysis. This technique has been proven useful for species identification and classification in the family *Enterobacteriaceae* (Mollet *et al.,* 1997; Drancourt *et al.,* 2001; Case *et al.,* 2006; Stephan *et al.,* 2007; Stephan *et al.,* 2008). 65 of the 470 isolates could not be identified (Popp *et al.,* 2009). The present study deals with the further characterization of five of these isolates and shows that they represent two novel species in the genus *Pantoea*.

The five isolates were obtained from powdered infant formula (1400/07^T, A11/07, A18/07^T) and the infant formula production environment (484/07, 1378/07) using the following approach. After enrichment of the samples by incubation for 24 h at 37 °C in buffered peptone water (BPW), 0.1 ml was transferred in 9 ml *Enterobacteriaceae* enrichment broth (Becton Dickinson, Spacks, MD, USA) and incubated for another 24 h at 37 °C. The obtained culture was thereafter plated on violet red bile glucose agar (Becton Dickinson, Spacks, MD, USA), incubated for 24 h at 37 °C and the five isolates were recovered as typical red colonies.

API ID32E analysis (bioMérieux, Marcy L'Etoile, France) was performed according to the manufacturer's instructions. The results suggested an identification as *Pantoea* spp. *1, Pantoea* spp. *3* as well as *Serratia plymuthica*, but with low levels of confidence. In a next step, partial *rpoB* gene sequences were determined following the procedure described by Mollet *et al.* (1997). Comparison of the partial *rpoB* gene sequences of the isolates amongst each other, suggested two distinct groups, with isolates 1400/07^T, A11/07, 484/07 and 1378/07 showing 99 to 100 % *rpoB* gene sequence similarity amongst each other on the one hand, and isolate A18/07^T showing 97 % *rpoB* gene sequence similarity with the other 4

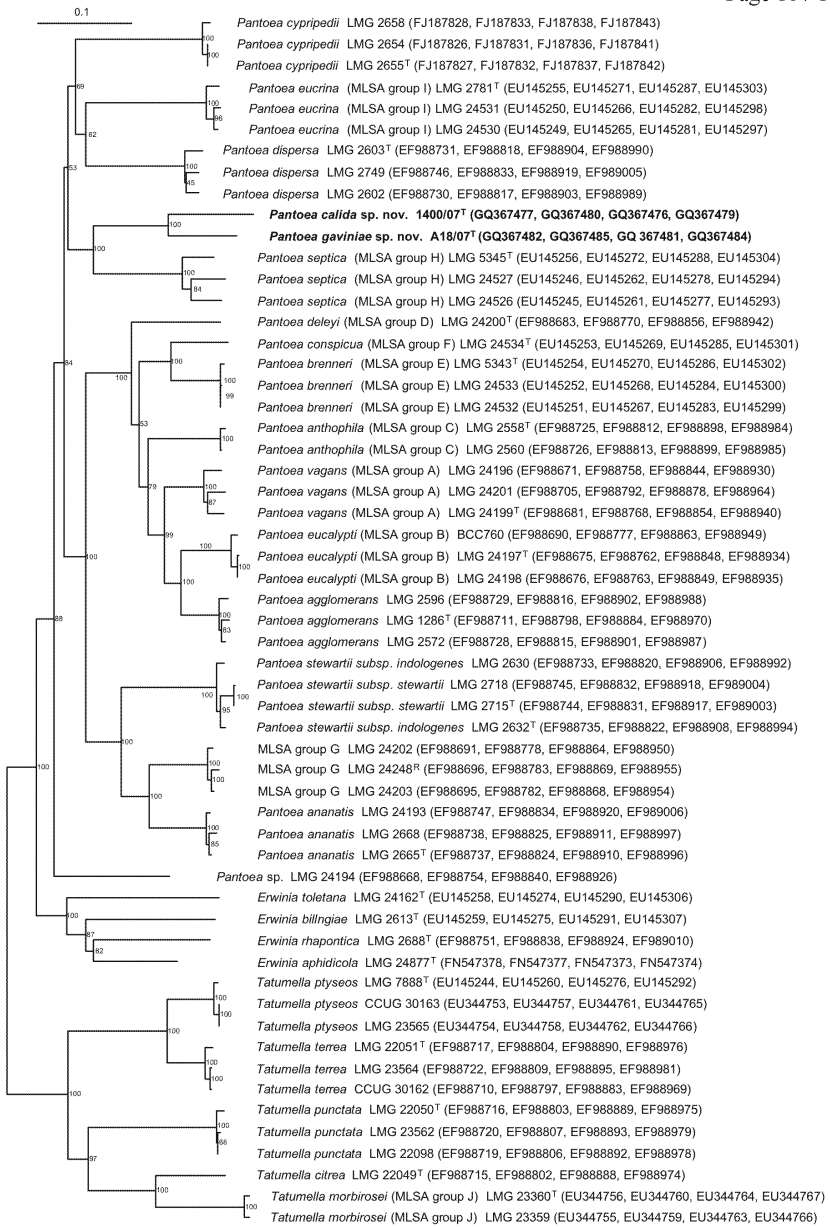


Figure 1: Neighbour-joining dendrogram based on concatenated partial *atpD, gyrB*, *infB* and *rpoB* gene sequences of *P. gaviniae* sp. nov., *P. calida* sp. nov. and related taxa within the family *Enterobacteriaceae*. The type strains are indicated with ^{T}. The reference strains of MLSA groups reported by Brady et al. (2008) are indicated with ^{R}. The scale bar indicates 10 % nucleotide substitutions. Bootstrap values (percentages of 500 replicates) are shown.

Table 1: Phenotypic characteristics (in grey) that differentiate *P. gaviniae* sp. nov. and *P. calida* sp. nov. fror

Taxa: 1, *Pantoea gaviniae* sp. nov. (n=1); 2, *Pantoea calida* (n=4); 3, *Pantoea brenneri;* 4, *Pantoea cons eucrina;* 7, *Pantoea cypripedii;* 8, *Pantoea vagans;* 9, *Pantoea eucalypti; 10, Pantoea deleyi; 11, Pantoea* 13, *Pantoea dispersa;* 14, *Pantoea ananatis;* 15, *Pantoea stewartii subsp. stewartii;* 16, *Pantoea stewartii sut* -, 0-10% positive; (-), 10-20% positive; v, 20-80% positive; (+), 80-90% positive; +, 90-100% positive; nd, n For taxa 3-7 indol production and fermentation data were generated within this study, carbon source utiliz data for taxa 8-11 are from Brady *et al.*, 2009b, data for taxa 12, 13 from Gavini *et al.*, 1989 and data for taxa

Characteristic	1	2	3	4	5	6	7	8	9	10
Indol production	–	–	–	–	–	–	–	–	–	–
Fermentation of										
Potassium-2-ketogluconate	+	+	+	+	+	+	+	nd	nd	nd
D-Arabitol	–	–	–	+	+	+	–	nd	nd	+
D-Lactose	+	+	–	+	–	–	+	+	+	nd
β-Gentiobiose	+	+	+	+	–	–	–	nd	nd	+
Potassium-5-ketogluconate	–	+	–	–	–	+	+	nd	nd	nd
D-Melibiose	+	+	–	–	–	–	+	nd	nd	nd
D-Sorbitol	–	+	–	–	–	–	–	nd	nd	nd
Kalium-gluconate	+	+	–	+	+	+	+	nd	nd	nd
Carbon source utilization										
β-Gentiobiose	+	+	–	+	nd	nd	+	+	–	+
4-Aminobutyrate	–	–	nd	nd	nd	nd	nd	nd	nd	nd
D-Raffinose	+	+	nd	–	nd	–	–	–	–	–
α-Ketoglutarate	+	+	nd	nd	nd	nd	nd	–	–	–
D-Arabitol	–	–	nd	+	nd	+	+	nd	nd	+
L-Tartrate	–	–	nd	+	nd	–	nd	+	+	+

Fermentation data (API 50CHE) were recorded after 48h. Data for carbon source utilization tests (Biotyp 100

isolates on the other hand. Additional phenotypic tests (API 20E and API 50CHE tests (bioMérieux, Marcy L'Etoile, France)) were performed in a next step, and the same two groups could be delineated based on the fermentation of galactonurate, sorbitol and Potassium-5-ketogluconate, and the utilization of citrate within 24 hours (positive for isolates 1400/07^T, A11/07, 484/07 and 1378/07, negative for A18/07^T).

In the present study, the phylogenetic position of the isolates based on partial *rpoB* gene sequences was investigated, since an MLSA scheme for the genus *Pantoea* based on partial *atpD, gyrB*, *infB* and *rpoB* gene sequences was recently reported (Brady *et al.*, 2008). Using the software package BioNumerics (Applied Maths, Belgium), the *rpoB* gene sequences of the isolates were compared to those of reference strains of *Pantoea* and related taxa (Brady *et al*. (2008) or newly determined sequences, i. e. *Erwinia aphidicola* LMG 24877^T). Phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987, Supplementary Fig. 1), maximum parsimony and maximum likelihood (Felsenstein, 1985) methods. The robustness of the branches was evaluated by bootstrap resamplings of the data (Felsenstein, 1985, Supplementary Fig. 1). The MP and ML trees showed basically the same topology as the NJ tree (data not shown). The new *rpoB* gene sequence analysis revealed that the five isolates formed two groups closely related with several *Pantoea* spp. and *Erwinia* spp., which indicated a phylogenetic position in the genus *Pantoea* or the genus *Erwinia*.

To refine the taxonomic position of the isolates, two representatives (A18/07^T and 1400/07^T), were subjected to multilocus sequence analysis of concatenated partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences, since Brady *et al*. (2008) reported that a concatenated data set tree based on these four gene sequences is to be preferred over trees based on single gene sequences for that purpose. In their concatenated data set trees, *Pantoea* species were supported by higher bootstrap values. Total DNA for *atpD*, *gyrB* and *infB* sequence analysis

was prepared according to the protocol of Niemann *et al.* (1997), and partial fragments of the *atpD*, *gyrB* and *infB* genes were amplified and sequenced following the protocol of Brady *et al*. (2008), except for 1400/07^T where the following forward and reverse primers were used to sequence the *gyrB* gene fragment: gyrB 07-F, gyrB 05-R (5'-TCGGAAGAAACCAGTTTGTCTT-3') and gyrB 08-R. Sequence assembly was performed using the program AutoAssembler™ (Applied Biosystems, Foster City, CA, USA), and phylogenetic analysis was performed using the software package BioNumerics (Applied Maths, Belgium). The partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences of the isolates were concatenated and aligned with concatenated partial *atpD*, *gyrB*, *infB* and *rpoB* gene sequences of reference strains of *Pantoea* and related taxa (Brady *et al*. (2008) or newly determined, i. e. *Erwinia aphidicola* LMG 24877^T). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987, Fig. 1) and maximum-parsimony (Felsenstein, 1985) methods, and the robustness of the branches was evaluated by bootstrap resamplings of the data (Felsenstein, 1985). The MP tree showed basically the same topology as the NJ tree and the ML tree by Brady *et al.* (2008) (data not shown). The multilocus sequence analysis revealed that isolates A18/07^T and 1400/07^T represented probably two novel species in the genus *Pantoea*, phylogenetically closely related to *Pantoea septica* (Brady *et al*., 2009a). Isolates A18/07^T and 1400/07^T were therefore further characterized.

The 16S rRNA genes of both isolates were amplified and sequenced as described by Franz *et al*. (2006), using the primers listed by Coenye *et al*. (1999). Almost complete 16S rRNA gene sequences comprising 1494 (A18/07^T) and 1495 (1400/07^T) nucleotides were obtained, and compared to almost complete 16S rRNA gene sequences of reference strains of *Pantoea* and related taxa (Brady *et al*., 2008 or newly determined sequences i. e. *Erwinia aphidicola* LMG 24877^T and *Erwinia toletana* LMG 24162^T). Pairwise similarities were calculated with the BioNumerics software package (Applied Maths, Belgium) using an open gap penalty of 100

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% and a unit gap penalty of 0 %. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), and the robustness of the branches was evaluated by bootstrap resamplings of the data (Felsenstein, 1985, Fig. 2). Isolates A18/07^T and 1400/07^T showed the highest 16S rRNA gene sequence similarity with *Pantoea deleyi* LMG 24200^T (98.0 and 97.9 %, resp.), *Pantoea conspicua* LMG 24534 (97.9 and 98.0 %, resp.), *Pantoea brenneri* LMG 5343 (97.8 and 97.9 %, resp.), *Pantoea cypripedii* ICMP 1591^T (97.8 and 97.7 %, resp.), *Erwinia toletana* LMG 24162^T (97.8 and 98.0 %, resp.), *Erwinia tasmaniensis* Et1/99^T (97.7 % each), *Pantoea septica* LMG 5345 (97.7 and 97.5 %, resp.), *Pantoea* MLSA group G LMG 24248 (97.7 % each), *Pantoea vagans* LMG 24199^T (97.7 and 97.9 %, resp.), *Pantoea agglomerans* NCTC 9381^T (97.6 and 97.8 %, resp.), *Pantoea eucalypti* LMG 24197^T (97.6 % each), *Pantoea anthophila* LMG 2558^T (97.5 and 97.7 %, resp.), *Pantoea ananatis* ATCC 33244^T (97.5 and 97.4 %, resp.), *Pantoea dispersa* LMG 2603^T (97.4 and 97.8 %, resp.), *Erwinia aphidicola* LMG 24877^T (97.4 % each), *Erwinia persicina* LMG 11254^T (97.2 and 97.3 %, resp.), *Enterobacter cowanii* CIP 107300^T (97.1 and 97.4 %, resp.), *Pantoea eucrina* LMG 2781 (97.1 and 97.0 %, resp.), and *Pantoea stewartii* subsp. *stewartii* LMG 2715^T (97.0 and 97.2 %, resp.). The 16S rRNA gene sequence similarity between isolates 1400/07^T and A18/07^T was 99.6 %. Overall, no 16S rRNA gene sequence similarities above 98.7-99 % were found with any of the validly described species, suggesting again that the isolates represented novel species within the family *Enterobacteriaceae* (Stackebrandt & Ebers, 2006).

Since the branches representing the different genera of the *Enterobacteriaceae* are not monophyletic and not supported by high bootstrap values and since the highest similarity values with the strains are found with reference strains of different genera, it can be concluded that for the *Enterobacteriaceae*, the obtained similarity levels reflect a high level of homoplasy in the 16S rDNA sequences.

DNA-DNA hybridizations were performed between isolates A18/07^T and 1400/07^T, and the type strains of the phylogenetically closest relatives based on comparative 16S rRNA sequence analysis. DNA for DNA-DNA hybridizations was prepared according the method of Wilson (1987), with minor modifications (Cleenwerck et al., 2002), and hybridizations were carried out at 45 °C using a modification (Goris et al., 1998; Cleenwerck et al., 2002) of the microplate method described by Ezaki *et al.* (1989). The DNA relatedness percentages reported are the means of minimum 7 hybridizations. Reciprocal reactions (i.e. AxB and BxA) were performed and the variation between them was within the limits of this method (Goris *et al.*, 1998). DNA-DNA hybridizations revealed 59 % (SD ± 4) DNA-DNA relatedness between isolates 1400/07^T and A18/07^T, and less than 25 % DNA-DNA relatedness between isolate 1400/07^T and the selected reference strains: *P. deleyi* LMG 24200^T (14 %, SD ± 5), *P. conspicua* LMG 24534^T (15 %, SD ± 1), *P. brenneri* LMG 5343^T (15 %, SD ± 6), *P. cypripedii* LMG 2657^T (14 %, SD ± 5), *E. toletana* LMG 24162^T (16 %, SD ± 14), *E. tasmaniensis* LMG 25318^T (15 %, SD ± 4), *P. septica* LMG 5345^T (23 %, SD ± 4), *P. vagans* LMG 24199^T (15 %, SD ± 4), *P. agglomerans* LMG 1286^T (18 %, SD ± 1), *P. eucalypti* LMG 24197^T (14 %, SD ± 3), *P. ananatis* LMG 2665^T (14 %, SD ± 0), *P. dispersa* LMG 2603^T (18 %, SD ± 13), *P. eucrina* LMG 2781^T (15 %, SD ± 7) and *P. stewartii* subsp. *stewartii* LMG 2715^T (8 %, SD ± 6). These values are below 70 %, the generally accepted limit for species delineation (Wayne *et al.,* 1987), demonstrating that the two isolates represented two novel genospecies.

The overall DNA G+C content of isolates A18/07^T and 1400/07^T was determined from DNA prepared in the frame of the DNA-DNA hybridizations, according to the HPLC method desribed by Mesbah et al. (1989). The values (means of three independent analyses of the same DNA sample) for A18/07^T and 1400/07^T were 58,4 and 57,4 mol%, respectively. These

The type strain 1400/07^T (= LMG 25383^T = DSM 22759^T) was isolated from powdered infant formula. The EMBL accession number for the 16S rRNA gene sequences of the type strain 1400/07^T is GQ367478. The EMBL accession numbers for the *atpD, gyrB*, *infB* and *rpoB* sequences of 1400/07^T are GQ367477, GQ367480, GQ367476 and GQ367479, respectively.

ACKNOWLEDGEMENTS

The BCCM/LMG Bacteria Collection is supported by the Federal Public Planning Service – Science Policy, Belgium. The authors wish to acknowledge Katrien Vandemeulebroecke, Leentje Christiaens and Katrien Engelbeen for their technical support.

galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine, arbutine, salicine, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-trehalose, β-gentiobiose, potassium-gluconate, potassium-2-ketogluconate, potassium-5-ketogluconate and galacturonate. **No acid production within 48 h under anaerobic conditions is observed from**: erythritol, D-arabinose, L-xylose, adonitol, methyl-β-D-xylopyranoside, L-sorbose, dulcitol, methyl-α-D-mannoyranoside, methyl-α-D-glucopyranoside, amygdaline, inuline, D-melezitose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. **Positive for the utilization of** α-D-glucose, β-D-fructose, D-galactose, D-trehalose, D-mannose, α-D-melibiose, saccharose, D-raffinose, maltotriose, maltose, α-lactose, lactulose, 1-0-methyl-β-galactopyranoside, D-cellobiose, β-gentiobiose, 1-0-methyl-β-D-glucopyranoside, esculin, D-ribose, L-arabinose, D-xylose, α-L-rhamnose, glycerol, myo-inositol, D-mannitol, D-sorbitol, D-saccharate, mucate, L-malate, cis-aconitate, trans-aconitate, citrate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, N-acetyl-D-glucosamine, D-gluconate, DL-lactate, succinate, fumarate, DL-glycerate, D-glucosamine, L-aspartate, L-glutamate, L-proline, D-alanine, L-alanine, L-serine and α-ketoglutarate within 4 days. **The following compounds are not utilized as sole carbon sources within 4 days:** L-sorbose, 1-0-methyl-α-galactopyranoside, palatinose, α-L-fucose, D-melezitose, D-arabitol, L-arabitol, xylitol, dulcitol, D-tagatose, maltitol, D-turanose, adonitol, hydroxyquinoline-β-glucuronide, D-lyxose, i-erythritol, 1-0-methyl-α-D-glucopyranoside, 3-0-methyl-D-glucopyranose, L-tartrate, D-tartrate, meso-tartrate, D-malate, tricarballylate, L-tryptophan, phenylacetate, protocatechuate, p-hydroxybenzoate, quinate, gentisate, m-hydroxybenzoate, benzoate, 3-phenylpropionate, m-coumarate, trigonelline, betain, putrescine, 4-aminobutyrate, histamine, caprate, caprylate, L-histidine, glutarate, 5-aminovalerate, ethanolamine, tryptamine, itaconate, 3-hydroxybutyrate, malonate, propionate and L-tyrosine.

DNA G+C content of the strain 1400/07^{T} is 57,4 mol%.

values are consistent with DNA G+C contents of other members of the genus *Pantoea* (Gavini *et al.*, 1989; Mergaert *et al.*, 1993, Brady *et al.,* 2009a, Brady *et al.,* 2009b).

The phenotypic characteristics of all five isolates from the various biochemical tests (API ID32E, API 20E, API 50CHE and Biotype 100 with Biotype Medium 1 (bioMérieux, Marcy L'Etoile, France), performed according to the manufacturer's instructions) were compared to characteristics of strains of the genus *Pantoea*. The phenotypic characteristics of the five isolates fit into the emended description of the genus *Pantoea* (Brady *et al.,* 2009a), and could be differentiated from the phylogenetically related *Pantoea* species by their ability to ferment D-lactose and utilize β-gentiobiose and D-raffinose, their inability to ferment and utilize D-arabitol and their inability to produce indol (Table 1). The two genospecies themselves could be differentiated, as previously mentioned, by fermentation of galactonurate, sorbitol and Potassium-5-ketogluconate (Table 1). Details on the physiological and biochemical characteristics of the novel species are given in the species descriptions below.

The results of this polyphasic taxonomic study support the recognition of two novel *Pantoea* species, for which the names *Pantoea gaviniae*, sp. nov. and *Pantoea calida,* sp. nov. are proposed.

Description of *Pantoea gaviniae*, sp. nov.

Pantoea gaviniae (ga.vi.ni'a.e. N. L. fem. gen. n. of Gavini, in honor of Françoise Gavini, a French microbiologist who first described the genus *Pantoea*).

Cells consist of Gram-negative rods that are facultative anaerobic and motile. Cells are 1.0 µm wide by 2.0 - 3.0 µm long and occur single or in pairs. After 24 h aerobic incubation at 37 °C on TSA medium, colonies are non-pigmented and convex. Oxidase-negative and catalase-

positive. The colonies grow well at 10 °C and poorly at 7 °C (within 3 days). They grow poorly at 41.5 °C but no growth occurred at 44 °C.

Positive for β-glucosidase, β-galactosidase, α-galactosidase, esculin hydrolysis and the Voges-Proskauer Test but **negative for** ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, urease, lipase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-glucosidase, α-maltosidase, L-aspartic acid arylamidase, tryptophane deaminase, gelatinase and indol and H_2S production. **Acid is produced under anaerobic conditions from the following compounds within 48 h:** glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, N-acetylglucosamine, arbutine, salicine, D-cellobiose, D-maltose, D-lactose, D-melibiose, saccharose, D-trehalose, D-raffinose, β-gentiobiose, potassium-gluconate and potassium-2-ketogluconate. **No acid production within 48 h under anaerobic conditions is observed for** erythritol, D-arabinose, L-xylose, adonitol, methyl-β-D-xylopyranoside, L-sorbose, dulcitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, amygdaline, inuline, D-melezitose, amidon, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium-5-ketogluconate and galacturonate. **Positive for the utilization of** α-D-glucose, β-D-fructose, D-galactose, D-trehalose, D-mannose, α-D-melibiose, saccharose, D-raffinose, maltotriose, maltose, α-lactose, lactulose, 1-0-methyl-β-galactopyranoside, 1-0-methyl-α-galactopyranoside, D-cellobiose, β-gentiobiose, 1-0-methyl-β-D-glucopyranoside, esculin, D-ribose, L-arabinose, D-xylose, α-L-rhamnose, glycerol, myo-inositol, D-mannitol, D-sorbitol, D-saccharate, mucate, L-malate, cis-aconitate, trans-aconitate, citrate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, N-acetyl-D-glucosamine, D-gluconate, DL-lactate, succinate, fumarate, DL-glycerate, D-glucosamine, L-aspartate, L-glutamate, L-proline, D-alanine, L-alanine, L-serine and α-ketoglutarate within 4 days. **The following compounds are not utilized as sole carbon sources within 4 days:** L-sorbose, palatinose, α-L-fucose, D-melezitose, D-arabitol, L-arabitol, xylitol, dulcitol, D-

tagatose, maltitol, D-turanose, adonitol, hydroxyquinoline-β-glucuronide, D-lyxose, i-erythritol, 1-0-methyl-α-D-glucopyranoside, 3-0-methyl-D-glucopyranose, L-tartrate, D-tartrate, meso-tartrate, D-malate, tricarballylate, L-tryptophan, phenylacetate, protocatechuate, p-hydroxybenzoate, quinate, gentisate, m-hydroxybenzoate, benzoate, 3-phenylpropionate, m-coumarate, trigonelline, betain, putrescine, 4-aminobutyrate, histamine, caprate, caprylate, L-histidine, glutarate, 5-aminovalerate, ethanolamine, tryptamine, itaconate, 3-hydroxybutyrate, malonate, propionate and L-tyrosine.

DNA G+C content of the strain A18/07^T is 58,4 mol%.

The type strain A18/07^T (= LMG 25382^T = DSM 22758^T) was isolated from powdered infant formula. The EMBL accession number for the 16S rRNA gene sequences of the type strain A18/07^T is GQ367483. The EMBL accession numbers for the *atpD, gyrB*, *infB* and *rpoB* sequences of A18/07^T are GQ367482, GQ367485, GQ367481 and GQ367484, respectively.

Description of *Pantoea calida*, sp. nov.

Pantoea calida (ca.li'da L. fem. adj. calida, meaning warm, hot, able to grow at 44°C).

Cells consist of Gram-negative, coccoid rods that are facultative anaerobic and motile. Cells are 1.0 µm wide by 1.5 - 2.0 µm long and occur single or in pairs. After 24 h aerobic incubation at 37 °C on TSA medium, colonies are non-pigmented and convex. Oxidase-negative and catalase-positive. The colonies grow poorly at 10 °C (within 3 days) but well at 44 °C.

P**ositive for** β-glucosidase, β-galactosidase, esculin hydrolysis and the Voges-Proskauer test but **negative for** ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, urease, lipase, β-glucuronidase, α-maltosidase, L-aspartic acid arylamidase, tryptophane deaminase, gelatinase and indol and H_2S production. **Acid is produced under anaerobic conditions from the following compounds within 48 h**: glycerol, L-arabinose, D-ribose, D-xylose, D-